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## SPECIAL ISSUE RESEARCH ARTICLE

# 6-Formylindolo[3,2-b]carbazole (FICZ) is a Very Minor Photoproduct of Tryptophan at Biologically Relevant Doses of UVB and Simulated Sunlight<sup>†</sup>

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## ABSTRACT

Exposure to solar UV is at the origin of numerous photodegradation pathways in biomolecules. Tryptophan is readily modified by UVB radiation into ring-opened and oxidized photoproducts. One of them, 6-formylindolo[3,2-b]carbazole (FICZ), has been extensively studied in the recent years because it very efficiently binds to AhR, a major factor in numerous biological processes, such as metabolism of xenobiotics. Unfortunately, little information is available on the actual yield of FICZ upon exposure to low and biologically relevant doses of UV radiation. In the present work, we used a sensitive and specific HPLC-tandem mass spectrometry assay to quantify a series of photoproducts induced by UVB and simulated sunlight (SSL) in solutions of tryptophan. FICZ represented only a minute amount of the photoproducts (0.02% and 0.03%, respectively). Experiments were repeated in culture medium where the yield of FICZ was also found to be very low, even when Trp was added. Last, no FICZ could be detected in cytosolic fractions of cultured cells exposed to SSL. Altogether, the present results show that FICZ is a very minor photoproduct and that it cannot be considered as the only endogenous photoproduct responsible for the induction of AhR-dependent responses in UV-irradiated cells.

## INTRODUCTION

Solar UV radiation exhibits a wide array of beneficial and deleterious effects in living systems. These biological responses arise from absorption of UV photons by endogenous chromophores. UVA (280-320 nm) photons are the most frequent (at least 95% of the incident UV radiation) but are the less energetic. The UVA-absorbing compounds are not extensively characterized, but include flavins, haems, and other cellular compounds (1). The UVB portion of the solar UV spectrum represents less than 5% of the incident photons but is the most energetic. UVB is absorbed by several important biomolecules. A well-known example is DNA, where UVB-induced excited states lead to the formation of mutagenic pyrimidine dimers (2). Another example is the 7-dihydrocholesterol which is

isomerized in skin by UVB and serves as a precursor of vitamin D (3). Proteins and aromatic amino acids are other major UV chromophores in cells (4).

Among the UV-absorbing amino acids, tryptophan (Trp) has been one of the most extensively studied (5). Trp is an essential amino acid required for many biological processes in human being but synthesized only by plants and micro-organisms. Its UV absorption spectrum, explained mostly by the presence of an indole moiety, peaks at 280 nm and spreads to 300 nm. Its molar absorption coefficient is 3-times larger than that of tyrosine, another important aromatic amino acid. Early studies have provided structural information of the major Trp photoproducts produced by photolysis under aerated conditions (Scheme 1). N-Formylkynurenine (NFKyn) has been identified as the major photoproduct, either following direct absorption of UVB or the singlet-oxygen mediated formation of an oxetane intermediate undergoing further rearrangement and ring opening (6, 7). Kynurenine (Kyn) is then produced from NFKyn by decarbonylation. Another important photolysis product of Trp is tryptamine (Trypta) produced via a decarboxylation reaction (6). Interestingly, several of these UV-induced photoproducts are also produced by the Trp metabolizing enzymes in the chain of biochemical process leading to major biological factors, such as melatonin, serotonin, or vitamin B3 (5). Trp also plays a key role in photoactive proteins. An example is DNA repair enzymes photolyases where a triad of Trp residues is involved in the photo-reduction of a flavin co-factor essential for the catalytic process (8).

More complex photoproducts of Trp have been also isolated and identified. Among them, 6-formylindolo[3,2-b]carbazole (FICZ) has received major attention (Scheme 1). One main reason is that FICZ was found to exhibit a very high affinity for the aryl hydrocarbon receptor (AhR) transcription factor (9, 10). AhR is a ligand-dependent transcription factor. Planar aromatic ligands like 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and polycyclic aromatic hydrocarbons bind to AhR

and promote its translocation to the nucleus where it induces the expression of numerous genes. They include the cytochrome P450 oxygenases involved in xenobiotics detoxification. Interestingly, the affinity constant of FICZ for AhR is 0.44 nM, similar to that of TCDD (0.48 nM) (10). As a consequence, FICZ has been proposed to account for most of the AhR-mediated pathways triggered by UV exposure, including modulation of immune responses, skin pigmentation, and induction of the metabolism of xenobiotics (11). In particular, FICZ and UV-irradiated tryptophan were shown to mimic the UV-induction of cytochrome P450 metabolizing enzymes in cultured HaCaT cells (12, 13) and hepatocytes (14). More recently, FICZ was also shown to behave as a UVA photosensitizer (15). It should still be emphasized that FICZ has only been directly observed in UVB-irradiated when cells were preliminary incubated with 1 mM Trp, a concentration that is 15- to 20-times more than can be found in classical culture media (12). The only evidence of the *in vivo* formation of FICZ is the detection of some of its metabolites in human urine, but without a clear link to UV exposure (16). The proposal of a photobiological function of FICZ relies mostly on indirect experiments, where addition of sunlight- or UVB-irradiated Trp (solutions or medium supplemented with Trp) were found to induce AhR dependent-pathways.

The actual level of FICZ in UV-exposed cells and skin therefore remains a pending issue. First, Trp, the precursor of FICZ, is among the less common residues found in proteins and is thus expected to be present in low concentration in cells. Second, formation of FICZ, the mechanism of which is not clearly established, involves a bimolecular reaction and its probability of occurrence thus decreases in a quadratic way when the Trp concentration decreases. Consequently, it is expected to be low in a cellular context where the Trp concentration is low. Third, we recently found (von Koschimbahr *et al.*, submitted) a modest 4-fold increase of *CYP* induction in skin explants and normal human keratinocytes (NHK) exposed to simulated sunlight (SSL), compared to the 100-fold induction in the presence of benzo[a]pyrene (B[a]P). In addition, co-exposure to SSL and B[a]P led unexpectedly to a decrease by a factor of 2 of the 100-fold induction of CYP450 observed with B[a]P alone. A similar trend was observed for the level of DNA adducts to the B[a]P diol epoxide metabolite. These results

suggest that UV-induced endogenous AhR ligands are either weak binders or produced in low amounts since exogenous ligands efficiently compete for the binding to AhR. Photochemical data reported in the literature are not very helpful to estimate the amount of FICZ produced from Trp, since they often use a single, large dose of radiation. We thus undertook the present work in order to precisely determine the extent of formation of FICZ at low dose. For this purpose, we developed an assay for several Trp photoproducts, including FICZ, which was then applied to the determination of their yields of formation in aqueous solutions of Trp exposed to UVB or SSL. Additional experiments were performed in cultured medium and cells.

## MATERIALS AND METHODS

*Chemicals.* Trp was a Merck product. It was first used in solution in 10-times diluted PBS.

Experiments were also performed in keratinocyte serum free medium (KSFM) with or without added Trp. HPLC separations were performed with deionized water containing 0.01% formic acid and acetonitrile. Pure FICZ, Trypta and Kyn were purchased from Sigma-Aldrich. NFKyn was purified from a UVB-irradiated solution of Trp. The elution was monitored at 320 nm and the main peak collected. The combined fractions were freeze-dried and purified a second time with the elution monitored at 230 nm. Only one peak (> 95% purity) was observed. The corresponding compound was identified as NFKyn on the bases of the MS/MS and UV absorption features. The concentration of the final solution was determined from the value of the absorption at 321 nm ( $\epsilon=3750 \text{ M}^{-1} \text{ cm}^{-1}$ ) (17).

*HPLC-MS/MS analyses.* HPLC-MS/MS analyses were performed on an API 3000 triple quadrupole mass spectrometer used in the positive electrospray ionization mode (SCIEX) interfaced with an Agilent 1000 series micro HPLC system. The fragmentation mass spectra of Trp, NFKyn, Kyn, Trypta and FICZ were recorded at different collision energy. The settings were then optimized in order to reach maximum sensitivity for the production of the major fragments which were then used for the

sensitive and specific detection with the mass spectrometer used in the multiple reaction monitoring mode (MRM). The following transitions were used:

Trp:  $m/z$  205 ( $MH^+$ )  $\rightarrow$  188 ( $MH^+ - NH_3$ )

NFKyn:  $m/z$  237 ( $MH^+$ )  $\rightarrow$  220 ( $MH^+ - NH_3$ );  $m/z$  237  $\rightarrow$  136 ( $MH^+ - CO - C_2H_3NO_2$ )

Kyn:  $m/z$  209 ( $MH^+$ )  $\rightarrow$  192 ( $MH^+ - NH_3$ )

Trypta:  $m/z$  161 ( $MH^+$ )  $\rightarrow$  144 ( $MH^+ - NH_3$ ) and  $m/z$  161 ( $MH^+$ )  $\rightarrow$  117 ( $MH^+ - NH_3 - HCN$ )

FICZ:  $m/z$  285 ( $MH^+$ )  $\rightarrow$  257 ( $MH^+ - CO$ )

An HPLC gradient was optimized to achieve a proper separation of Trp and its 4 photoproducts. It consisted in an increasing proportion of acetonitrile in 0.05 % mass spectrometry grade formic acid (ACN: 0 min: 5%, 10 min 5% 30 min 80% 40 min 95%). On these bases, an HPLC-MS/MS method was built. The quantitative aspect of the detection was based on the determination of external calibration curves built with repeated injection of solution of pure standards of known concentrations.

*UV irradiation of Trp solutions.* Trp was first used in solution (10 mM) in 10-times diluted PBS (final concentration 13.7 mM NaCl, 0.3 mM KCl, 1 mM  $Na_2HPO_4$ , 0.2 mM  $KH_2PO_4$ ). A first series of irradiations were performed with a 2x15 W UVB lamp exhibiting an emission spectrum centered at 312 nm. Irradiations were performed in 1 cm optical path quartz cell (3 ml). Samples were exposed to UVB for increasing periods of time in triplicates. The corresponding doses were 0.06, 0.11, 0.22, 0.56, 1.1, 2.2 and 3.3  $J/cm^2$ . A similar protocol was used for exposure to SSL. The main difference was that irradiation of a glass petri dish (liquid height 1 cm) was performed from above. The applied doses ranged from 0.5 to 12 minimal erythemal doses (MED) of SSL, corresponding to UV exposures of 1.8, 3.6, 10.8, 21.6 and 43.2  $J/cm^2$ . After collection, the solutions were placed into injection vials

and frozen until HPLC-MS/MS analysis. Another series of experiments were performed in keratinocytes culture medium (KFSM). First, KFSM was exposed to two doses of SSL. In another experiment, 1 mM Trp was added to KFSM and irradiated. A control experiment was made with 1 mM Trp in 10-times diluted PBS.

*Cellular experiments.* Keratinocytes obtained from fresh human skin samples (18) were grown in keratinocytes serum-free medium (KFSM) containing 25 µg/mL bovine pituitary extract (BPE), 0.9 ng/mL of recombinant human epidermal growth factor (EGF), and 1% Pen/Strep. BPE and EGF were removed from the cell culture media 24 hours before irradiation. Four MEL501 derived melanoma lines were used. Two of them expressed AhR. The two other did not express, as the results of gene silencing by the CRISPR/Cas9 technology. MEL501 cells were grown in RPMI medium containing L-glutamine and 1% Pen/Strep. Non-decomplemented fetal calf serum (10 %) was added. All cells were grown at 37°C in 5% CO<sub>2</sub>. Before irradiation, keratinocytes and MEL501 were rinsed twice with PBS, and exposed to 2 or 10 MED SSL in PBS, respectively. Un-irradiated controls were also collected. Cell pellets were immediately collected and frozen at -20°C. They were then suspended in a lysis buffer (10 mM Hepes solution (pH 7.9), 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, and 0.01% (v/v) Triton X-100 in water). They were incubated for 2 hours on ice and vortexed every 30 minutes. The completion of the cellular lysis was checked by a trypan blue exclusion assay under a microscope. After lysis, extracts were passed through 0.2 µm cut-off filters and transferred into HPLC vials.

## RESULTS AND DISCUSSION

### *HPLC-MS/MS analysis of Trp photoproducts.*

Trp and the four photoproducts, FICZ, Kyn, NKyn, Trypta, provided fragmentation spectra with a few daughter ions. As expected, the relative intensity of the ions on the fragmentation spectra depended



on the applied collision energy (Fig. 1). Optimization of the MS/MS parameters and of the HPLC conditions provided a method for the detection of the photoproducts. A good sensitivity was reached for NFKyn, Trypta, and FICZ. In contrast, the limit of detection was 10 times higher for Kyn (Fig. 2).

### ***Photolysis of Trp by UVB radiation***

A first experiment involved exposure of a Trp solution to UVB. This wavelength range was chosen because it represents the portion of solar spectrum best absorbed by Trp. Irradiations were performed under aerated conditions since Trp photochemistry is dominated by photooxidation reactions (6) and because cellular photodynamic processes are known to be oxygen-dependent (1). A large number of data is available on the photophysics and final products of photolysis of Trp under anaerobic conditions (6). We did not investigate such irradiation conditions because the cellular environment contains oxygen and because formation of FICZ involves oxidization products of tryptophan. The UVB dose range was selected to be in the linear part of the dose-course formation. Indeed, we observed that use of very large doses leads to secondary photoreactions and the readily consumption of initial photoproducts (data not shown). Using HPLC-MS/MS, the four targeted photoproducts were detected in the irradiated Trp solutions, although at very different levels (Fig. 3). In particular, the intensity of the peaks corresponding to NFKyn and FICZ were in a 4 orders of magnitude ratio. Collection of quantitative data by external calibration allowed us to calculate the yields of each photoproduct (Table 1). Based on these vales, the proportion of FICZ among the photoproducts was found to be 0.02%

### ***SSL-induced degradation of Trp***

In a following step, we investigated the formation of the same photoproducts upon exposure to SSL. The use of the latter source is important. First, because it is obviously more representative of actual

exposures than pure UVB. We also applied low SSL doses ranging between 0.5 and 12 MED. A second reason that motivates the use of SSL is that FICZ was first identified in samples of Trp exposed to the radiation emitted by a “black light”, the spectrum of which covered a wide range of wavelengths (9, 10). We thus wanted to check whether UVA applied simultaneously with UVB could affect the photochemistry of Trp and modulate the proportion of FICZ. Like for UVB, HPLC-MS/MS measurements allowed us to calculate the yield of formation of the Trp photoproducts (Table 1). FICZ was again found to be produced only in minute amounts, representing 0.03% of the analyzed photoproducts. NFKyn was by far the most abundant photoproduct. The dose-course formation of the photoproducts also provided interesting information. The formation was linear with respect to the applied SSL dose for NFKyn, Kyn, and Trypta, showing that these compounds are primary photodegradation products (Fig. 4). In contrast, a quadratic shape was observed for FICZ (Fig. 4), which suggests that this compound is either a secondary photoproduct or arises from a bimolecular reaction between primary photoproducts. The mechanism of formation of FICZ is not clearly understood, but was proposed to involve condensation of 2 molecules of 3-( $\alpha$ -hydroxyacetaldehyde)-indole (10), a degradation product of 3-( $\alpha$ -acetaldehyde)-indole (19). Our observations are therefore in agreement with the fact that FICZ is not a direct Trp photolysis product. Consequently, its yield will be low at low concentration of Trp and at low UV doses.

#### ***Degradation of Trp in culture medium***

Some previously published works showed that exposure of culture medium to artificial and natural solar light was shown to produce FICZ (20). These results led us to repeat our UV exposure using SSL in KFSM, the medium used for our keratinocyte experiments. When pure medium, that regularly contains 64  $\mu$ M Trp, was irradiated, the four photoproducts were detected. NFKyn was still the major photoproduct (Table 2), but in lower proportion than when 10 mM Trp in PBS was exposed to UVB and SSL. The proportion of FICZ was higher in pure medium (0.3%) than in the experiments

carried out at 10 mM in PBS. Yet, this may be explained by the decreased contribution of NFKyn, whose formation may be modulated by components of the culture medium. The latter solution contains vitamins exhibiting reducing properties and may thus alter the fate of intermediates in photooxidation reactions. In addition, other components like antibiotics absorb UV photons and may behave as photosensitizers. This may also affect the distribution of the Trp photoproducts. Irradiation of culture medium where 1 mM pure Trp was added led to yields of formation of the four photoproducts like those observed in a 1 mM solution of Trp in 10-times diluted solution (Table 2). One exception was FICZ, the concentration of which was below the detection limit in the irradiated medium spiked with 1 mM Trp. This is a surprising observation since exposure of pure medium led to the formation of FICZ. It should be stressed that in the latter case, the detected amount is very low (approximately 30 fmol in 20  $\mu$ l of medium exposed to the largest dose), which is very close to the basal level of FICZ in the Trp solution (25 fmol in 20  $\mu$ l after dilution in the medium). It is thus likely that the UV-induced amount of FICZ is low in the Trp-spiked medium and not distinguishable from the background. This part of the work shows that irradiation in culture medium leads to the formation of Trp photoproducts, NFKyn being again the major photoproduct and FICZ a very minor one.

#### ***Trp photoproducts in cells exposed to SSL***

In order to complete the quantitative results of these photochemical studies, we analyzed cytosolic extracts of cells exposed to SSL where the amount of free Trp and its photoproducts was determined by HPLC-MS/MS. We investigated primary normal human keratinocytes (NHK) and four cell lines derived from human-originated cutaneous malignant melanoma (MEL501). In both control and irradiated cells, a higher content in Trp was observed in the MEL501 cell lines (160 pmol/ $10^6$  cells) compared to NHK (30 pmol/ $10^6$  cells). The four studied Trp photoproducts were quantified in both control and irradiated cells. A basal level (0.8 pmol/ $10^6$  cells) of NFKyn was detected in HNK. This

value was increased by a factor 3 following exposure to 2 MED, the conditions used in our previous study (von Koschembahr *et al.* submitted). None of the three other photoproducts were detected, in particular, FICZ (Fig. 5). In MEL501 cell lines, none of the Trp derivatives were detected neither in unirradiated cells nor following exposure to 10 MED. This could not be accounted for by an analytical problem associated with the presence of the lysis buffer, since the photoproducts were readily detected in samples spiked with authentic standard (Fig. 6 for FICZ). Absence of FICZ was observed in the 4 MEL501 cell lines, although two of them lacked the AhR protein following CRISPR/Cas9 silencing. The lack of AhR was expected to prevent the degradation of FICZ and favor its detection. Based on the sensitivity of the HPLC-MS/MS, a lower limit for the amount of FICZ produced by SSL is 3 fmol /  $10^6$  cells. This value would correspond to a yield of 0.003% with respect to the initial intracellular amount of Trp, compatible with the results of the photochemical study. Altogether, these results confirm the conclusion of a very low yield of FICZ upon photolysis of Trp, even in a cellular context. These results contrast with a previous report of the formation of FICZ in HaCaT cells (12). However, the latter involved a rather large dose of UVB, whereas we used SSL. In addition, cells were preincubated with 1 mM Trp, whereas we used a classical medium with a concentration of 64  $\mu$ M in Trp. Interestingly, the authors calculated a formation yield of 0.0001% for FICZ, namely in the same low range than our upper estimation.

## CONCLUSIONS

The present observations provide some quantitative information of the yields of some major Trp photoproducts. Using either UVB or SSL, and for the latter exposure of 1 mM Trp in culture medium, NFKyn was always found to be the overwhelming major photoproduct produced in roughly 50- and 100-times larger yield than Kyn and tryptamine, respectively. When experiments were performed at 10 mM in aqueous solution, the FICZ represented only 0.02 to 0.03% of the photoproducts. This proportion was higher when pure medium was irradiated, but no significant formation of FICZ could

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be established in Trp-spiked medium. These observations, combined with the evidence of a non-linear dependency of FICZ with respect to the dose, allow us to conclude that FICZ is really a very minor photoproduct. This conclusion was strengthened by the lack of detection of FICZ in cells exposed to SSL. These results are critical to comprehend the effect of UV radiation on AhR-dependent pathways (11). Indeed, FICZ is the almost unique endogenous photoproduct proposed in the current literature as a mediator in such processes. This putative role of FICZ is challenged by our negative results in SSL-exposed cells, although the very high affinity of FICZ for AhR (10) may partly compensate its low intracellular concentration. However, the possible involvement of endogenous photoproducts other than FICZ in the AhR-mediated effect of UV radiation have to be considered. Our present results show that other Trp derivatives are produced in much larger yield than FICZ. Moreover, Trp and amino acids are not the only source of endogenous photoproducts. Therefore, there is still a need for identifying other candidates arising from the photolysis of various biomolecules as mediators of the UV-induced AhR activation.

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## TABLES

**Table 1.** Yield of formation of Trp photoproducts following exposure to either UVB or simulated sunlight (SSL). Results are expressed in % of formations with respect to the initial amount of Trp per  $\text{kJ cm}^{-2}$ . The errors are the standard error of the slopes ( $n=8$  and  $n=6$  for UVB and SSL, respectively).

	FICZ	NFKyn	Kyn	trypta
UVB	$0.023 \pm 0.002$	$95.1 \pm 4.1$	$8.44 \pm 0.19$	$0.862 \pm 0.054$
SSL	$0.010 \pm 0.002$	$31.2 \pm 0.6$	$1.00 \pm 0.04$	$0.038 \pm 0.001$

**Table 2.** Yield of formation of Trp photoproducts in culture medium. Pure medium was exposed, as well as medium spiked with 1 mM Trp. A control experiment was performed with 1 mM Trp in solution in 10-times diluted PBS. Results are expressed in % of formation with respect to the initial amount of Trp per  $\text{kJ cm}^{-2}$ . Trp concentrations of 0.064, 1, and 1.064 were used for calculation in medium, PBS and Trp-spiked medium. The errors are the standard error of the slopes.

	FICZ	NFKyn	Kyn	trypta
Pure medium	$0.077 \pm 0.031$	$19.1 \pm 4.1$	$6.92 \pm 0.77$	$1.54 \pm 0.33$
1 mM Trp in diluted PBS	$0.13 \pm 0.02$	$23.4 \pm 2.6$	$0.48 \pm 0.04$	$0.15 \pm 0.03$
1 mM Trp in medium	Not detected	$24.9 \pm 0.9$	$0.85 \pm 0.06$	$0.67 \pm 0.03$



## FIGURES AND SCHEME LEGENDS

**Scheme 1.** Chemical structure of the main Trp photoproducts. Kyn: Kynurenine, NFKyn: N-Formylkynurenine, Trypta: tryptamine, FICZ: 6-Formylindolo[3,2-b]carbazole.

**Figure 1.** Fragmentation mass spectra of some Trp photoproducts. a) Fragmentation spectrum of NFKyn recorded with a collision energy (CE) of 59 eV and b) effect of the collision energy on the intensity of the pseudo-molecular ion ( $m/z=285.2$ ) and the  $[MH]^+-CO$  fragment ( $m/z=257$ ) of FICZ

**Figure 2.** HPLC-MS/MS detection of 20 pmol of Kyn, NFKyn, Trypta and FICZ. The HPLC-MS/MS system was operated in the MRM mode with a gradient of acetonitrile in a 0.01% aqueous solution of formic acid.

**Figure 3.** HPLC-MS/MS detection of photoproducts in photolyzed Trp. The overall UVB dose applied to the analyzed sample was  $3.3 \text{ J cm}^{-2}$ . The identity of the photoproducts was inferred from their HPLC retention times and their mass spectrometry features (Kyn  $m/z$  209  $\rightarrow$  162; NFKyn  $m/z$  237  $\rightarrow$  220 and 136; Trypta  $m/z$  161  $\rightarrow$  144 and 117; FICZ  $m/z$  285  $\rightarrow$  257).

**Figure 4.** Dose-course formation of Trp photoproducts upon exposure to SSL. Results are expressed in % of formations with respect to the initial amount of Trp. Data are the mean  $\pm$  standard error (n=3).

**Figure 5.** Formation of Trp photoproducts in NHK exposed to 2 MED of SSL. The figure shows HPLC-MS/MS chromatograms. NFKyn was unambiguously identified on the basis of the retention time of

the peak and the presence of 2 specific daughter ions ( $m/z$  237 $\rightarrow$ 220 and 237 $\rightarrow$ 136). No peak was detected for FICZ at the expected retention time (33.7 min).

**Figure 6.** Quantification of FICZ in MEL501 cells exposed to 10 MED. The HPLC-MS/MS chromatogram corresponds to a) cytosolic extracts from irradiated MEL501 cells, b) standard solution of pure FICZ, and c) cytosolic extracts from non-irradiated MEL501 spiked with 200 fmol of FICZ. MRM transitions were  $m/z$  237  $\rightarrow$  220 and 136 for NFKyn and  $m/z$  285  $\rightarrow$  257 for FICZ.





